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(54) Title: FLUORESCENT PROTEINS FROM NON–BIOLUMINISCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF

(57) Abstract

The present invention is directed to novel fluorescent proteins from non–bioluminiscent organisms from the Class Anthozoa. Also disclosed are cDNAs encoding the fluorescent proteins.

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FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF

BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This is a divisional application of U.S.S.N. 09/210,330 filed on December 11, 1998.

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, cDNAs encoding the proteins and uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps, however, make the process laborious and difficult to control. An

alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β-galactosidase, firefly luciferase and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets in a compact structure, where, in the center, an alpha helix containing

the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

The present invention is directed to DNA sequences encoding fluorescent proteins selected from the group consisting of:

(a) an isolated DNA from an organism from the Class Anthozoa which encodes a fluorescent protein; (b) an isolated DNA which hybridizes to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code and that encodes a fluorescent protein. Preferably, the DNA is isolated from a non-bioluminescent organism from Class Anthozoa. More preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55, 57 and 61, and the fluorescent protein has the amino acid sequence shown in SEQ ID No. 56.

In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising the DNA and regulatory elements necessary for expression of the DNA in the cell. Preferably, the DNA encodes a fluorescent protein having the amino acid sequence shown in SEQ ID No. 56.

In still another embodiment of the present invention, there is provided a host cell transfected with a vector of the present invention, such that the host cell expresses a fluorescent protein. Preferably, the cell is selected from the group consisting of bacterial cells, mammalian cells, plant cells and insect cells.

The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) isolated DNA from an organism from Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code, and which

encodes a fluorescent protein. Preferably, the protein has the amino acid sequence shown in SEQ ID No. 56.

The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of: (a) an isolated DNA which encodes a fluorescent protein, wherein the DNA is from an organism from Class Anthozoa and wherein the organism does not exhibit bioluminescence; (b) an isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Actiniaria. More preferably, the organism is from Sub-order Endomyaria. Even more preferably, the organism is from Family Actiniidae, Genus Anemonia. Even more preferably, the organism is *Anemonia majano*. Most particularly, the present invention is drawn to a novel fluorescent protein from *Anemonia majano*, amFP486.

The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridizaton, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers). In the case of *Anemonia majano*, the first degenerate primer used was NGH (SEQ ID No. 4), and the second degenerate primer used was GNG(b) (SEQ ID No. 10).

Figure 2 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 3 shows transient expression of pCNFPMut32-N1 and pECFP-N1 in 293 cells, respectively. PCNFPMut32-N1 (**Figure 3A**) shows brighter fluorescent intensity and less photobleaching compared to pECFP-N1 (**Figure 3B**). pCNFPMut32-N1 is constructed by amplifying Mut32 DNA and then inserting the amplified product into EGFP-N1 backbone.

Figure 4 shows that fusion protein PKC-γ-CNFP translocated from cytosol to the plasma membrane when cells were treated with PMA (Phorbol 12-Myristate 13-Acetate). **Figure 4A** shows the result from control (without the treatment) and **Figure 4B** shows the result from PMA-treated cells.

Figure 5 shows functional analysis of destabilized amFP486. **Figure 5A** shows that expression of pCNFP-MODCd1 in HEK 293 cells exhibited purple fluorescence (pseudocolor). However, the actual color should be cyan (control). **Figure 5B** shows that transient transfection of pCNFP-MODCd1 demonstrates 50% decreased fluorescent intensity after 4-hour treatment with protein synthesis

inhibitor cycloheximide. pCNFP-MODCd1 is constructed using Mut32 DNA clone.

Figure 6 shows histogram of wildtype amFP486. The fluorescent intensity of the transfected cells was analyzed on FACS using FL1 (510/30) detecting channel. Five samples (**A-E**) were analyzed in parallel. Geo Mean = geometric mean for data points.

Figure 7 shows histogram of Mut15. Same method as in Figure 5 was used. Five samples (**A-E**) were analyzed in parallel.

Figure 8 shows histogram of Mut32. Same method as in Figure 5 was used. Five samples (**A-E**) were analyzed in parallel.

Figure 9 shows the expression of fusion protein Mut15-mdm2 in HEK293 cells.

Figure 10 shows the spectrum of wildtype amFP486. EX = 458 nm, EM = 492 nm, both slits = 2.5 nm.

Figure 11 shows the spectrum of Mut15.

Figure 12 shows the spectrum of Mut32.

Figure 13 shows the spectra of wildtype and mutant amFP486 on the same graph.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al.,

Nature 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

As used herein, the term "CFP" refers to cyan fluorescent protein, and the term "ECFP" refers to enhanced cyan fluorescent protein.

As used herein, the term "NFP" refers to novel fluorescent protein, and the term "CNFP" refers to cyan novel fluorescent protein. Specifically, "CNFP" refers to amFP486.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not

limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5'

direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when

the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: gluetamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: metionine; N: asparagine; P: proline; Q: gluetamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophane; Y: tyrosine; X: any residue). NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-59 is used.

The present invention is directed to an isolated DNA selected from the group consisting of: (a) isolated DNA from an organism from the Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the

degeneracy of the genetic code, and which encodes a fluorescent protein. Preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55, 57 and 61, and the fluorescent protein has the amino acid sequence shown in SEQ ID No. 56. More preferably, the DNA is amFP486, Mut15 or Mut32, or humanized version.

In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising the DNA and regulatory elements necessary for expression of the DNA in the cell. Specifically, the DNA encodes a fluorescent protein having the amino acid sequence shown in SEQ ID No. 56. Preferably, the vector is constructed by amplifying the DNA and then inserting the amplified DNA into EGFP-N1 backbone, or by fusing different mouse ODC degradation domains such as d1, d2 and d376 to the C-terminal of the DNA and then inserting the fusion DNA into EGFP-N1 backbone.

In still another embodiment of the present invention, there is provided a host cell transfected with the vector of the present invention, which expresses a fluorescent protein of the present invention. Preferably, the cell is selected from the group consisting of bacterial cells, mammalian cells, plant cells and insect cells. A representative example of mammalian cell is HEK 293 cell and an example of bacterial cell is an *E. coli* cell.

The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of: (a) an isolated DNA which encodes a fluorescent protein, wherein the DNA is from an organism from Class Anthozoa and wherein the organism does not exhibit bioluminescence; (b) an isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of

(a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Actiniaria. More preferably, the organism is from Sub-order Endomyaria. Even more preferably, the organism is from Family Actiniidae, Genus Anemonia. Most preferably, the organism is *Anemonia majano.*

The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) an isolated protein encoded by a DNA which encodes a fluorescent protein wherein the DNA is from an organism from Class Anthozoa and wherein the organism does not exhibit bioluminescence; (b) an isolated protein encoded by a DNA which hybridizes to isolated DNA of (a); and (c) an isolated protein encoded by a DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to degeneracy of the genetic code. Preferably, the isolated and purified fluorescent protein is amFP486.

The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridizaton, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16 and is used as a primer in polymerase chain reaction. Alternatively, it can be used as a probe for hybridization screening of the cloned genomic or cDNA library.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Biological Material

Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

TABLE 1

Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp. "green"	Western Pacific	green spots on oral disk
Anemonia sulcata	Mediterranean	purple tentacle tips

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EXAMPLE 2

cDNA Preparation

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthetized starting with 1-3 μg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG$(T)_{13}$, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 μM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 μl of this dilution was used in subsequent procedures.

TABLE 2

Oligos Used in cDNA Synthesis and RACE

TN3: 5'-CGCAGTCGACCG$(T)_{13}$
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCAGTCGACCG$(T)_{13}$
(SEQ ID No. 17)

TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

T7-TS:
5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

EXAMPLE 3

Oligo Design

To isolate fragments of novel fluorescent protein cDNAs, PCR using degenerate primers was performed. Degenerate primers were designed to match the sequence of the mRNAs in regions that were predicted to be the most invariant in the family of fluorescent proteins. Four such stretches were chosen (Table 3) and variants of degenerate primers were designed. All such primers were directed to the 3'-end of mRNA. All oligos were gel-purified before use. Table 2 shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used for Isolation of Fluorescent Proteins

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7)
	GEGNG (SEQ ID No. 8)	GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4

Isolation of 3'-cDNA Fragments of nFPs

The modified strategy of 3'-RACE was used to isolate the target fragments (see Figure 1). The RACE strategy involved two consecutive PCR steps. The first PCR step involved a first degenerate primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3' portion identical to the TN3 primer used for cDNA synthesis (for sequence of T7-TN3, Table 2). The reason for substituting the longer T7-TN3 primer in this PCR step was that background amplification which occurred when using the shorter TN3 primer was suppressed effectively, particularly when the T7-TN3 primer was used at a low concentration (0.1 _M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-9002). The second PCR step involved the TN3 primer (SEQ ID No. 1, Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

The first PCR reaction was performed as follows: 1 µl of 20-fold dilution of the amplified cDNA sample was added into the reaction mixture containing 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate

primer (Table 4) and 0.1 μM of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μl of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μM dNTPs, 0.3 μM of the second degenerate primer (Table 4) and 0.1 μM of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5

Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel fluorescent protein cDNAs, two nested 5'-directed primers were synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were then amplified using two consecutive PCRs. In the next PCR reaction, the novel approach of "step-out PCR" was used to suppress background amplification. The step-out reaction mixture contained 1x Advantage KlenTaq Polymerase Mix using buffer provided by the manufacturer (CLONTECH), 200 μM dNTPs, 0.2 μM of the first gene-specific primer (see Table 5), 0.02 μM of the T7-TS primer (SEQ ID No. 18), 0.1 μM of T7 primer (SEQ ID No. 19) and 1 μl of the 20-fold dilution of the amplified cDNA sample in a total volume of 20 μl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was diluted 50-fold in water and one μl of this dilution was added to the second (nested) PCR. The reaction contained 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 200 μM dNTPs, 0.2 μM of the second gene-specific primer and 0.1 μM of TS primer (SEQ ID No. 2) in a total volume of 20 μl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 12 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was then cloned into pAtlas vector (CLONTECH) according to the manufacturer's protocol.

TABLE 5

Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'-GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of NFPs in *E.coli*

To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 6). Primers with SEQ ID Nos. 36 and 37 were the primers used to prepare the am486 DNA. Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and NFP. The PCR was performed as follows: 1 µl of the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of upstream primer and 0.2 µM of downstream primer, in a final total volume of 20 µl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium

(supplemented with 100 μg/ml of ampicillin) at 37°C overnight. 100 μl of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 μg/ml of ampicillin and grown at 37°C, 200 rpm up to OD_{600} 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nFPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctctttcaaaca agtttatc (SEQ ID No. 36) BamHI	5'-tagtactcgagcttattcgta tttcagtgaaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacatttttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcaaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgaggttggaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttatc (SEQ ID No. 43) BamHI	5'-tagtactcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttccttttttaaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgttccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgaggccattacg ctaatc (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtgcacttaaagaagaaatg (SEQ ID No. 51)	5'-tagtactcgagattcggtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7

Novel Fluorescent Proteins and cDNAs Encoding the Proteins

One of the full-length cDNAs encoding novel fluorescent proteins is described herein (amFP486). The nucleic acid sequence and deduced amino acid sequence are SEQ ID Nos. 55 and 56, respectively. The spectral properties of amFP486 are listed in Table 7, and the emission and excitation spectrum for amFP486 is shown in Figure 2.

TABLE 7

Spectral Properties of the Isolated amFP486

Species:	Anemonia majano	Max. Extinction Coefficient:	40,000
nFP Name:	amFP486	Quantum Yield	0.24
Absorbance Max. (nm):	458	Relative Brightness:*	0.43
Emission Max. (nm):	486		

*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 8

Construction of amFP486 Mutants

Two mutants of amFP486 were generated, Mut15 and Mut32. Mut15 has the nucleic acid sequence shown in SEQ ID No. 57. Compared with wildtype amFP486, Mut15 has the following point

mutations: A to G at position 101 (numbered from beginning of ATG); T to C at position 129; AAA to TTG at positions 202-204; C to T at position 240. Table 8 lists the spectral properties of Mut15 and Mut32.

TABLE 8

Spectral Properties of the Isolated Mut15 and Mut32

Species:	Anemonia majano	Max. Extinction Coefficient:	53,400
nFP Name:	Mut15	Quantum Yield	0.32
Absorbance Max. (nm):	460	Relative Brightness:*	0.78
Emission Max. (nm):	485		
Species:	Anemonia majano	Max. Extinction Coefficient:	36,000
nFP Name:	Mut32	Quantum Yield	0.42
Absorbance Max. (nm):	466	Relative Brightness:*	0.69
Emission Max. (nm):	488		

*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 9

Construction and Functional Analysis of Vectors

Mut32 DNA was amplified via PCR and reconstructed to EGFP-N1 backbone with BamHI and NotI restriction enzyme sites. This

vector has the same multiple cloning sites as EGFP-N1. The nucleic acid sequence of the vector (pCNFPMut32-N1) is shown in SEQ ID No. 58.

Functional test of the generated vectors was performed by transient transfection in 293 cells. After 24-hour expression, brighter fluorescent intensity and less photobleaching of pCNFPMut32-N1 were observed by microscopy when compared with pECFP-N1 side by side (Figures 3A and 3B).

Mut32 has fast folding and bright fluorescent intensity, which makes it useful for number of applications. Some fusion proteins were tested, such as PKC-gamma-CNFP. PKC was observed to translocate from cytosol to the plasma membrane when cells were treated with PMA (phorbol 12-myristate 13-acetate). Figure 4 shows control and PMA-treated cells.

EXAMPLE 10

Generation of Destabilized amFP486 Vectors as Transcription Reporters

Since amFP486 is very stable, it is necessary to generate destabilized versions of amFP486 in order to observe the rapid turnover of the protein. By using the same technology for destabilized EGFP, three destabilized amFP486 vectors were constructed by fusing different mouse ODC degradation domains such as d1, d2 and d376 to the C-terminal of wild type amFP486. The sequences for vectors pCNFP-MODCd1 and pCNFP-MODCd2 are shown in SEQ ID No. 59 and SEQ ID No. 60, respectively. The vectors were constructed in EGFP-N1 backbone.

Vectors of pCRE-d1CNFP and pNF-κB-d1CNFP were constructed by placing d1CNFP downstream of cAMP response element

(CRE) or NF-κB response element, respectively. Expression of d1CNFP is up-regulated upon activation of these response elements.

EXAMPLE 11

Functional Analysis of Destabilized amFP486

Functional test of the destabilized amFP486 was performed by transient transfection in 293 cells. After 24-hour expression, the fluorescent intensity was decreased gradually from d2, d1 and d376 because of the fusion with different mouse ODC degradation domains. After 4-hour treatment with protein synthesis inhibitor cycloheximide, d2 fluorescent intensity did not change very much; however, d1 fluorescent intensity decreased further 50% of its original intensity (Figures 5A and 5B). The half-life of d1 is around 4 hours.

MODCd1 is a valuable tool for application as a transcription reporter. However, compared with EGFP-d1 (1-hour half-life), pCNFP-MODCd1 half-life (4 hours) is still long, so further mutagenesis for MODC degradation domain is still needed for shorter half-life version.

Functional test of vectors pCRE-d1CNFP and pNF-κB-d1CNFP was performed by transient transfection in HEK 293 cells. 16 hours post transfection, 10 μm forskolin was added to induce CRE and 100 ng/ml TNF-alpha was added to induce NF-κB for 6 hours. Expression of d1CNFP was analysed using FACS Calibur. Up to 7 fold increase of fluorescence in forskolin induced CRE activation and 4 fold increase of fluorescence in TNF-alpha induced NF-KB activation was observed (data not shown).

EXAMPLE 12

Construction and Functional Test for Humanized Mut32 (phCNFP-N1)

Since mammalian expression is a very popular tool, human favored codon version is needed for better expression in mammalian cells. To generate humanized Mut32, the Mut 32 sequence was first changed to human favored codon and 23 oligos (12F and 11R) were designed. Next, four rounds of PCR amplification were performed, each round for 20 cycles. PCR cycle was designed as follows: 94°C for 1 min; 94°C for 1 min; 40°C for 1 min; and 72°C for 1 min. The four rounds were: for 1st round, mixing 2 µl each of every 4 oligos (60 bp), 5 µl buffer, 1 µl pfu, 1 µl dNTP to make total volume of 50 µl. After 20 cycles of PCR, 5 sets of 150 bp and 1 set of 4 last oligos of 90 bp products were obtained. For 2nd round, mixing new crude PCR products 10 µl each, 5 µl buffer, 1 µl pfu, 1 µl dNTP to make total volume of 50 µl. After 20 cycles of PCR, 2 sets of 270 bp and 1set of 210 bp PCR products were obtained. For 3rd round, mixing new crude PCR products. After 20 cycles of PCR, 1 set of 510 bp and 1 set of 450 bp products were obtained. For 4th round, mixing new crude products. After 20 cycles of PCR, final PCR product (690 bp) was obtained. Further PCR amplification was performed using 1F and 11R primers.

As a result, humanized Mut32 was generated, having the sequence shown in SEQ ID No. 61. This humanized Mut32 was constituted into EGFP-N1 backbone.

EXAMPLE 13

Expression of Wildtype and Mutant amFP486 in Mammalian Cells

The original plasmid amFP486 DNAs (wildtype, Mut15 and Mut32 in pQE30) were used to construct N1 version of amFP486 wildtype, Mut15 and Mut32 as described in Example 9. The DNAs were inserted into *E.coli* DH5α. HEK 293 cells were transferred with each of the three N1 constructs using Calcium Phosphate method (Clontech product #K2051-1).

The fluorescent intensity of the transfected cells was analyzed on FACS using FL1 (510/30) detecting channel. Five samples were analyzed in parallel for each construct. The histograms of all the analysis are shown in Figures 6-8.

The M1 gate is set as shown on the histograms. The mean value of FL1 fluorescent intensity of the M1 population of each sample is summarized in Table 9. It shows that the average of the mean value of each construct (Wildtype, Mut15, and Mut32) has no significant difference.

TABLE 9

FL1 Fluorescent Intensity of M1 Population

Sample #	Wildtype (Figure 6A-6E)	Mut15 (Figure 7A-7E)	Mut32 (Figure 8A-8E)
1	82.84	106.95	84.51
2	77.52	108.73	91.41
3	111.85	97.08	91.30
4	113.06	90.16	98.16
5	104.95	86.34	111.44
Mean	98.04	97.85	95.36

EXAMPLE 14

Generation and Expression of Fusion Protein Mut15-mdm2

The Mut15-mdm2 fusion was generated by the following steps: first, mdm2 DNA was obtained by amplifying human Marathon cDNA library (Burke's Lymphoma) using primers ATGTGCAATACCAACATGTCTGTACC (SEQ ID No. 62) and CTAGGGGA AATAAGTTAGCAC (SEQ ID No. 63); secondly, the purified PCR product was then amplified with primers GGAATTCCAGCCATGGTGTG CAATACCAACATGTCTGTACC (SEQ ID No. 64) and TCCCCCGGGGGGAA ATAAGTTAGCAC (SEQ ID No. 65) in order to add Kozac sequence and restriction sites; thirdly, the purified PCR product from step 2 was digested with EcoR I and Sma I and inserted into EcoR I and SmaI of NFP1Mut15-N1 vector (this vector was generated using BamH I and Not I sites of the pEGFP-N1 backbone).

The generated Mut15-mdm2 fusion was then expressed in HEK293 cells. Figure 9 shows the results.

EXAMPLE 15

Comparison of the Protein Fluorescent Intensity

PQE30 amFP486 wildtype, Mut15 and Mut32 were transformed into DH5α. The bacteria grew in the presence of 1 mM IPTG overnight to induce the protein expression. Cells were lysed in 100 mM Tris, pH8.0 by sonication. Cell lysate was collected after centrifuge at 3000 rpm for 15 minutes at room temperature. The proteins were purified with TALON Metal Affinity Resin. Briefly, after the protein was absorbed on the resin, the beads were washed in stepwise with first wash, then first elution (50 mM imidazole) and second elution (200 mM imidazole) in 100 mM Tris-HCl, pH 8.0. The protein is found mostly in the second step elution. It was found that Mut32 has the highest bacterial expression level, while Mut15 has the lowest.

Samples of each elution fraction were run on SDS-PAGE to check the purity of the proteins. Both wildtype amFP486 and Mut32 show a single band, while Mut15 has two more minor bands with higher molecular weight (data not shown).

The protein concentration (fractionII-2) was checked and measured by Bradford assay (Bio-Rad standard assay) using BSA as a standard. The spectra are shown in Figures 10-13. The fluorescence intensity (fraction II-2) was determined with LS50B Luminescence Spectrometer LS50B. EX = 458 nm, EM = 492 nm, both slits = 2.5 nm. Table 10 shows the protein concentration, relative fluorescent (FL)

intensity and intensity/μg protein in 700 μl volume. It shows that Mut32 is as bright as wildtype, while Mut15 is worse than the wildtype.

TABLE 10

	Protein Concentration	Relative FL Intensity	Intensity/μg Protein in 700 μl Volume
Wildtype II-2	1.26 μg/5 μl	37.805/5 μl	30.00
Mut15II-2	0.64 μg/5 μl	10.152/5 μl	15.86
Mut32II-2	6.17 μg/5 μl	186.474/5 μl	30.22

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects and ends inherent therein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein, are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes to the methods and compounds, and other uses, will occur to those skilled in the art and are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A DNA sequence encoding a fluorescent protein selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein, wherein said DNA is from an organism from a Class Anthozoa and wherein said organism does not exhibit bioluminescence;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

2. The DNA sequence of claim 1, wherein said organism is from Sub-class Zoantharia.

3. The DNA sequence of claim 2, wherein said organism is from Order Actiniaria.

4. The DNA sequence of claim 3, wherein said organism is from Sub-order Endomyaria.

5. The DNA sequence of claim 4, wherein said organism is from Family Actiniidae.

6. The DNA sequence of claim 5, wherein said organism is from Genus Anemonia.

7. The DNA sequence of claim 6, wherein said organism is *Anemonia majano.*

8. A DNA sequence encoding a fluorescent protein selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein having a nucleotide sequence selected from the group consisting of SEQ ID Nos. 55, 57, and 61;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code, and which encodes a fluorescent protein.

9. The DNA sequence of claim 8, wherein said DNA encodes a fluorescent protein having an amino acid sequence shown in SEQ ID No. 56.

10. The DNA sequence of claim 8, wherein said DNA is selected from the group consisting of amFP486, Mut15 and Mut32.

11. The DNA sequence of claim 8, wherein said DNA is humanized DNA.

12. A vector capable of expressing the DNA sequence of claim 1 in a recombinant cell, wherein said vector comprising said DNA and regulatory elements necessary for expression of the DNA in the cell.

13. The vector of claim 12, wherein said DNA encodes a fluorescent protein having the amino acid sequence shown in SEQ ID No. 56.

14. The vector of claim 12, wherein said vector is constructed by amplifying said DNA and then inserting the amplified DNA into EGFP-N1 backbone.

15. The vector of claim 14, wherein said DNA is selected from the group consisting of amFP486, Mut15 and Mut32.

16. The vector of claim 14, wherein said DNA is humanized DNA.

17. The vector of claim 12, wherein said vector is constructed by fusing different mouse ODC degradation domains to the C-terminal of said DNA and then inserting the fusion DNA into EGFP-N1 backbone.

18. The vector of claim 17, wherein said mouse ODC degradation domains are selected from the group consisting of d1, d2 and d376.

19. The vector of claim 17, wherein said DNA is selected from the group consisting of non-humanized and humanized DNA.

20. A host cell transfected with the vector of claim 12, wherein said cell is capable of expressing a fluorescent protein.

21. The host cell of claim 20, wherein said cell is selected from the group consisting of bacterial cells, mammalian cells, plant cell, yeast and insect cells.

22. The host cell of claim 21, wherein said mammalian cell is HEK 293 cell.

23. The host cell of claim 21, wherein said bacterial cell is an *E. coli* cell.

24. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein from an organism from Class Anthozoa, wherein said organism does not exhibit bioluminescence;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

25. The isolated and purified fluorescent protein of claim 24, wherein said organism is from Sub-class Zoantharia.

26. The isolated and purified fluorescent protein of claim 25, wherein said organism is from Order Actiniaria.

27. The isolated and purified fluorescent protein of claim 26, wherein said organism is from Sub-order Endomyaria.

28. The isolated and purified fluorescent protein of claim 27, wherein said organism is from Family Actiniidae.

29. The isolated and purified fluorescent protein of claim 28, wherein said organism is from Genus Anemonia.

30. The isolated and purified fluorescent protein of claim 29, wherein said organism is *Anemonia majano*.

31. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

(a) isolated DNA which encodes a fluorescent protein having an amino acid sequence shown in SEQ ID No. 56;

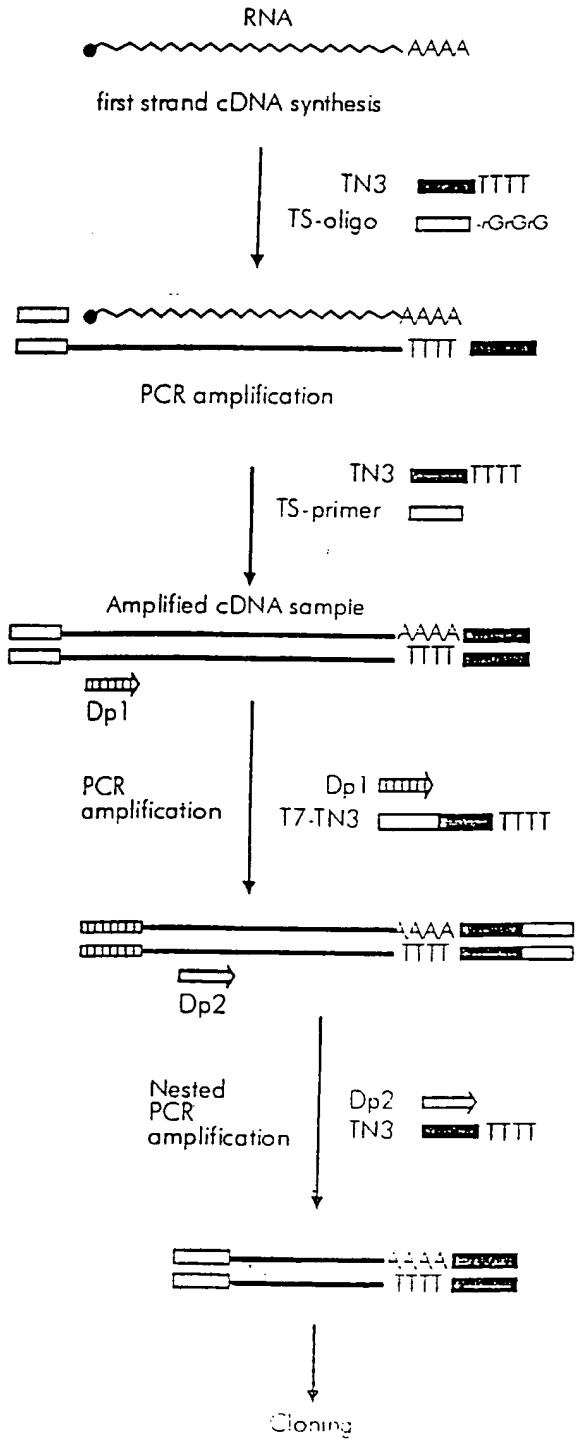
(b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) isolated DNA differing from said isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

32. The isolated and purified fluorescent protein of claim 31, wherein said protein is amFP486.

33. An amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridizaton, wherein said sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14.

34. The amino acid sequence of claim 26, wherein said oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16.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AAAA
TTTT
PCR amplification
TN3 TTTT
TS-primer
Amplified cDNA sample
AAAA
TTTT
Dp1
PCR amplification
Dp1
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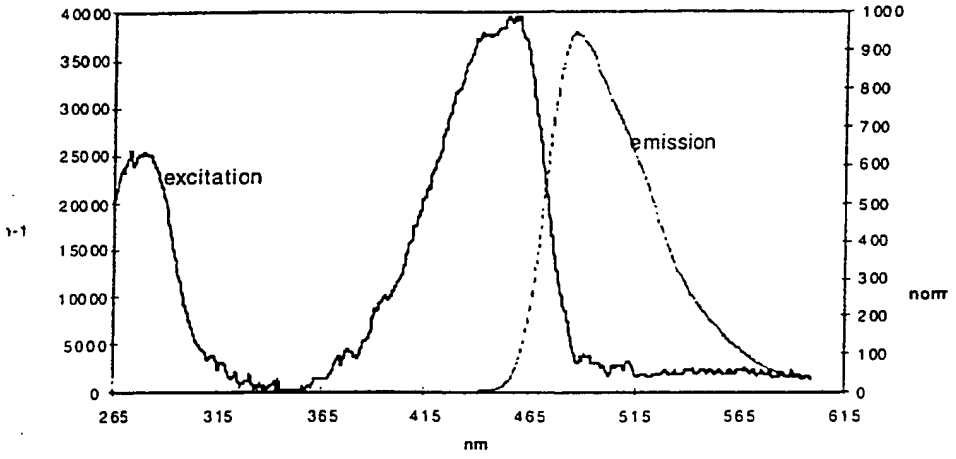
AAAA
TTTT
Dp2
Nested PCR amplification
Dp2
TN3 TTTT
AAAA
TTTT
Cloning

Figure 1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900
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315
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415
465
515
565
615
nm

Figure 2

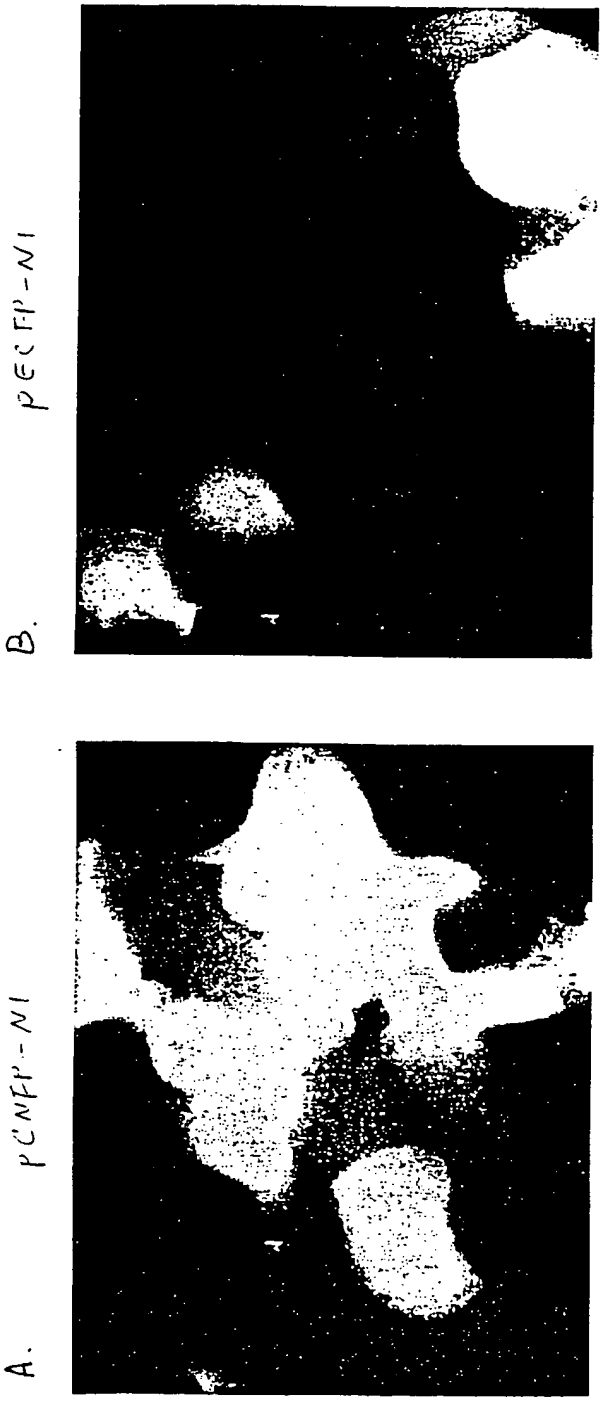


Figure 3

Traci Yerby,12/9/99 12:.. PM -0800,NFP1 Data

Date: Thu, 09 Dec 1999 12:17:31 -0800
From: "Traci Yerby" <TRYERBY@CLONTECH.COM>
To: <baadler@flash.net>
Subject: NFP1 Data
Mime-Version: 1.0

Hi Ben,
This should be the last of it.
Data for PMA treated cells (FIG 8)
Traci

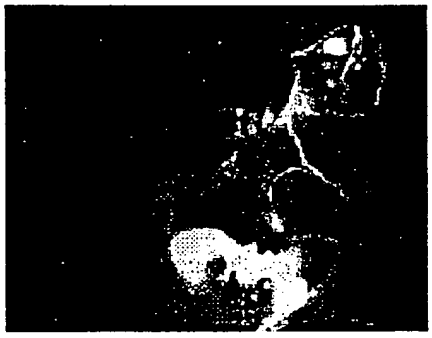
Content-Type: application/octet-stream; name="PKCr-NCFP.psd"
Content-Disposition: attachment; filename="PKCr-NCFP.psd"

PKCgamma-NCFP translocation

Fig. 4A

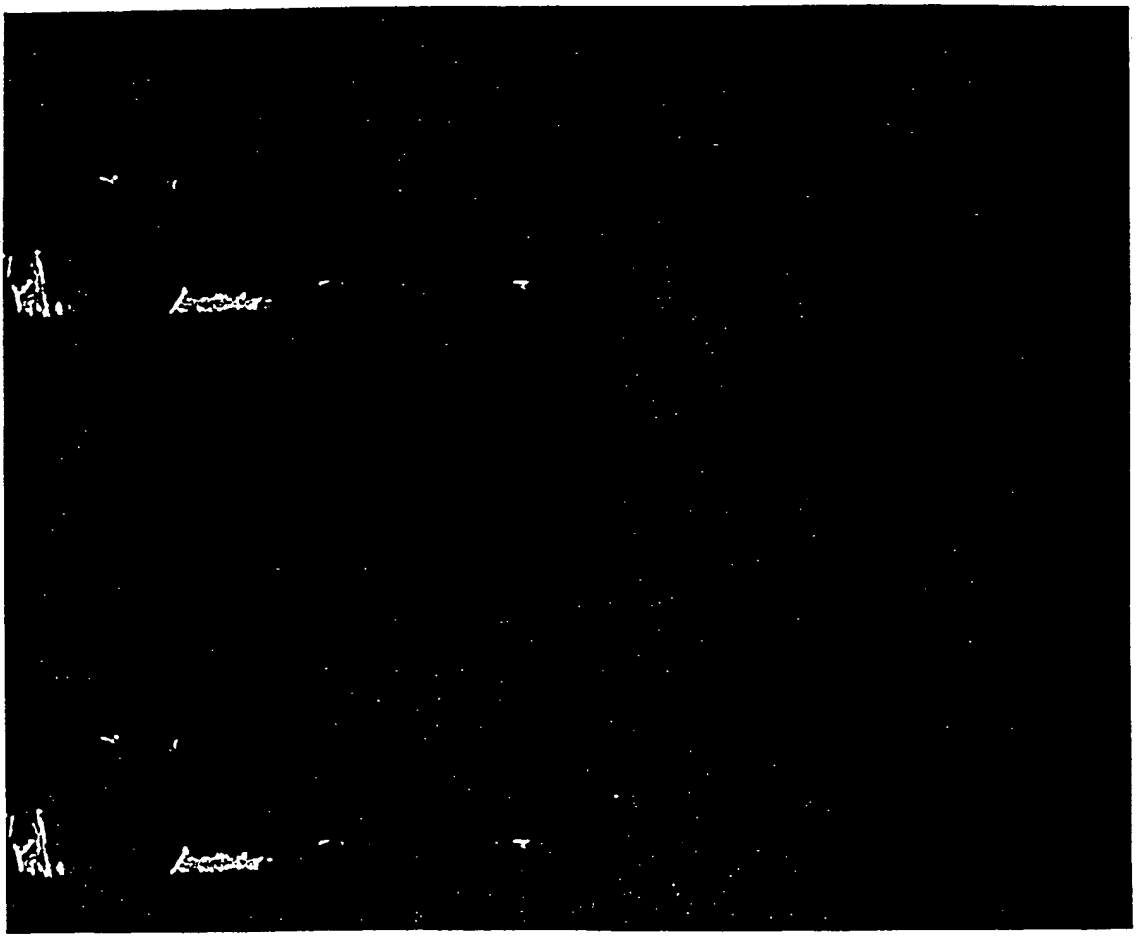
control

Fig. 4B



PMA treated

Figure 5A

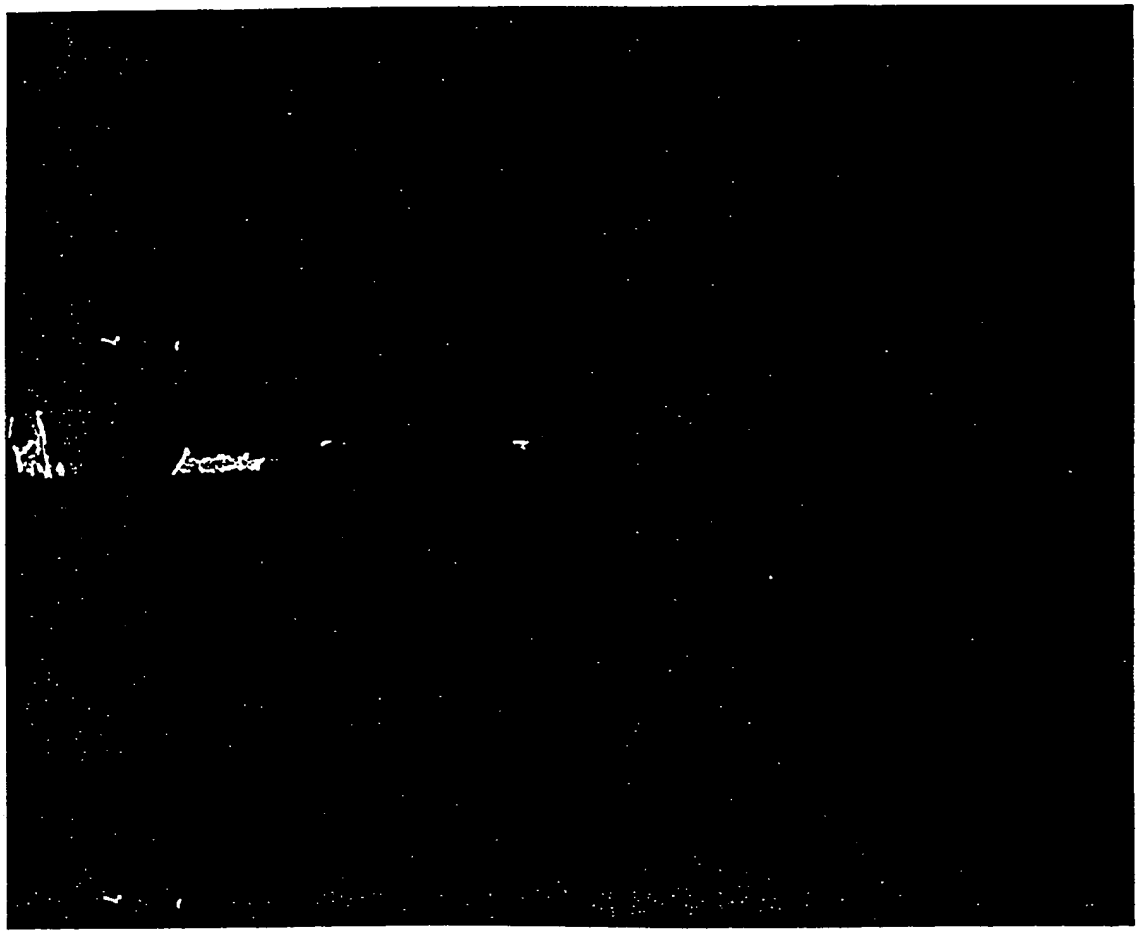


pCNFP-MUDC 11 control

picture 2

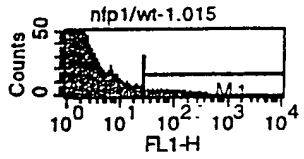
previous sample should be appr...

Figure 5B



PCNFP-MUDC(1) 4h + cycloheximide

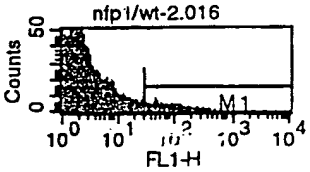
A.



File: nfp1/wt-1.015 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	5.19	2.13
M1	3.07	82.84	66.13

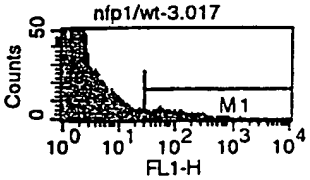
B.



File: nfp1/wt-2.016 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	5.49	2.17
M1	3.73	77.52	62.95

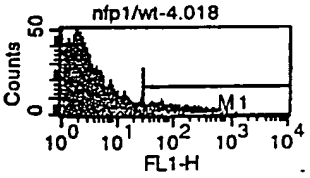
C.



File: nfp1/wt-3.017 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	7.75	2.31
M1	4.57	111.85	80.87

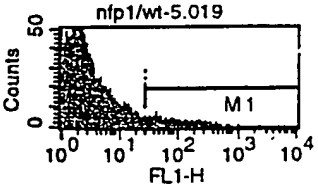
D.



File: nfp1/wt-4.018 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	8.56	2.43
M1	5.16	113.06	82.26

E.

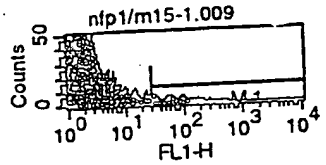


File: nfp1/wt-5.019 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.41	2.22
M1	3.61	104.95	77.54

Figure 6

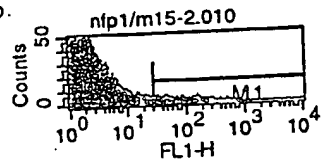
A.



File: nfp1/m15-1.009 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	5.74	2.17
M1	2.94	106.95	80.29

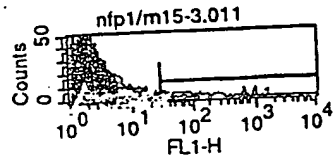
B.



File: nfp1/m15-2.010 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.57	2.24
M1	3.66	108.73	78.64

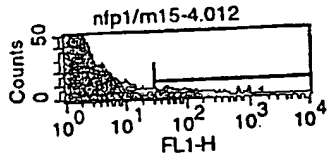
C.



File: nfp1/m15-3.011 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.26	2.24
M1	3.70	97.08	73.22

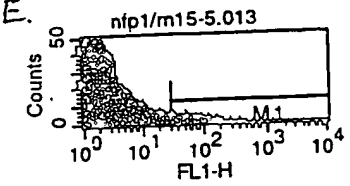
D.



File: nfp1/m15-4.012 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.83	2.28
M1	4.65	90.16	69.85

E.

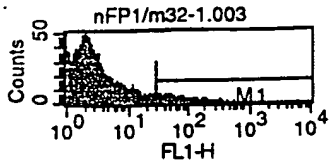


File: nfp1/m15-5.013 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.32	2.31
M1	4.16	86.34	67.43

Figure 7

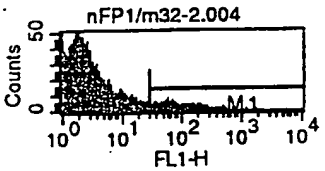
A.



File: nFP1/m32-1.003 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	5.55	2.08
M1	3.51	84.51	67.46

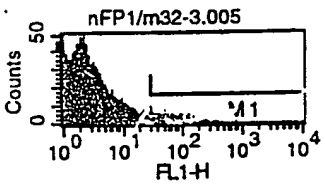
B.



File: nFP1/m32-2.004 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.30	2.13
M1	4.09	91.41	69.57

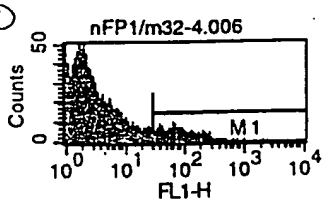
C.



File: nFP1/m32-3.005 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.44	2.20
M1	4.16	91.30	70.91

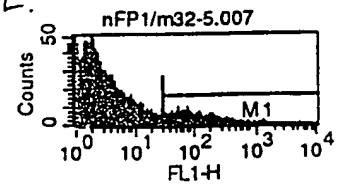
D



File: nFP1/m32-4.006 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	7.64	2.21
M1	5.15	98.16	71.62

E.



File: nFP1/m32-5.007 Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	10.07	2.41
M1	6.74	111.44	78.24

Figure 8

Fusion Mdm2 - nFP 1 mut 15

Figure 9

Date: 9/8/99 Time: 12:01:36 PM

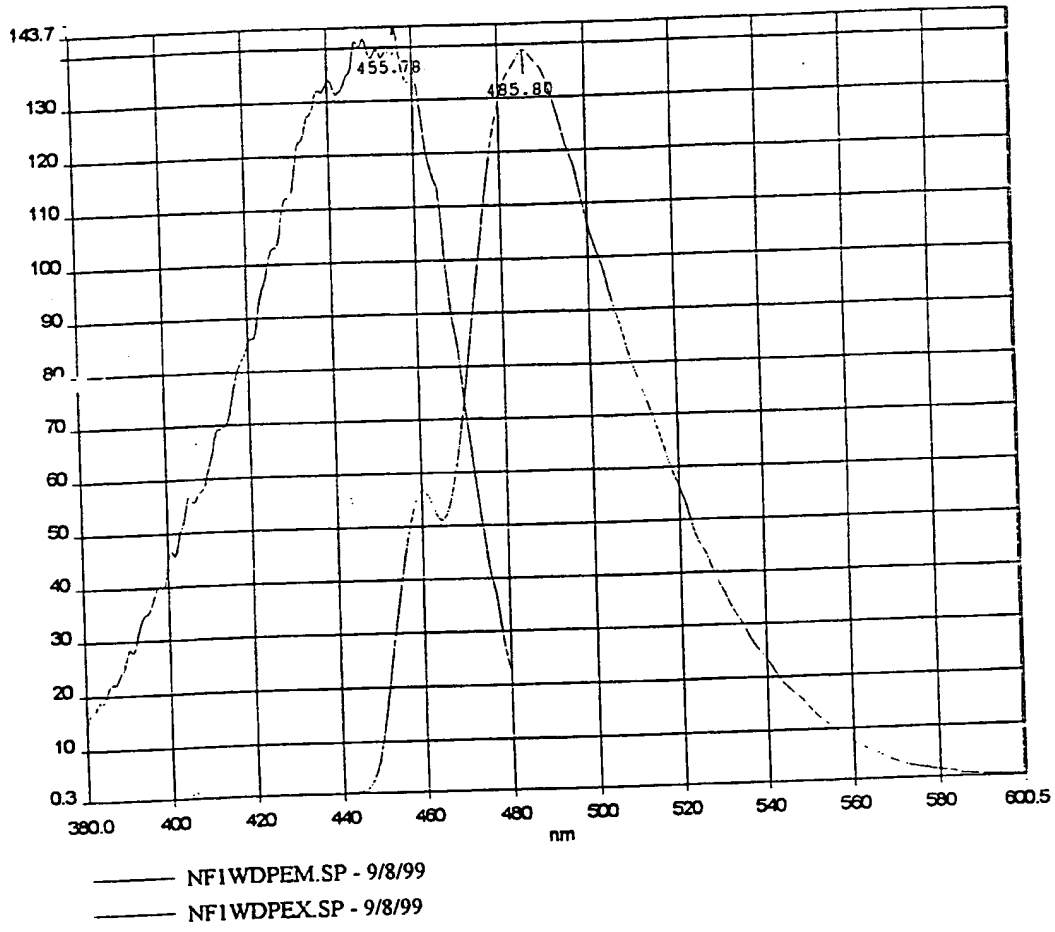


Figure 1D

Date: 9/8/99 Time: 12:05:19 PM

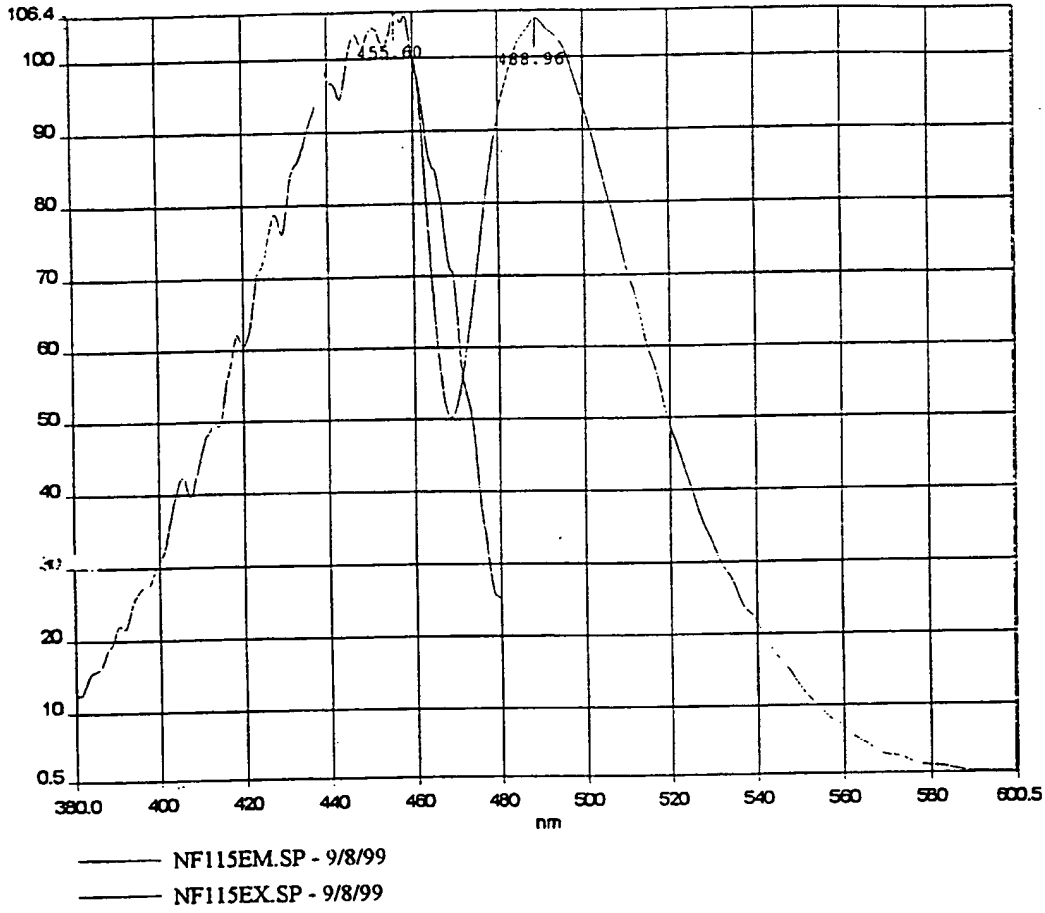


Figure 11

Date: 9/8/99 Time: 12:09:58 PM

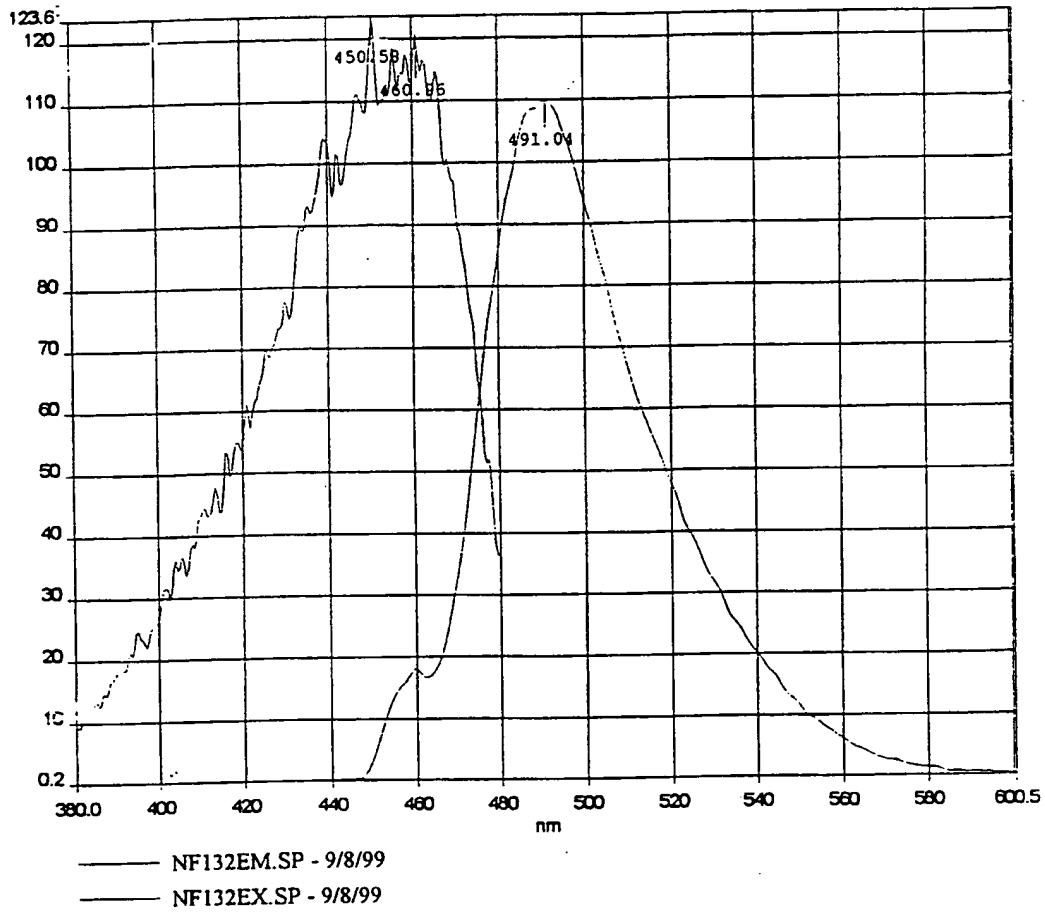


Figure 12

Date: 9/8/99 Time: 11:44:24 AM

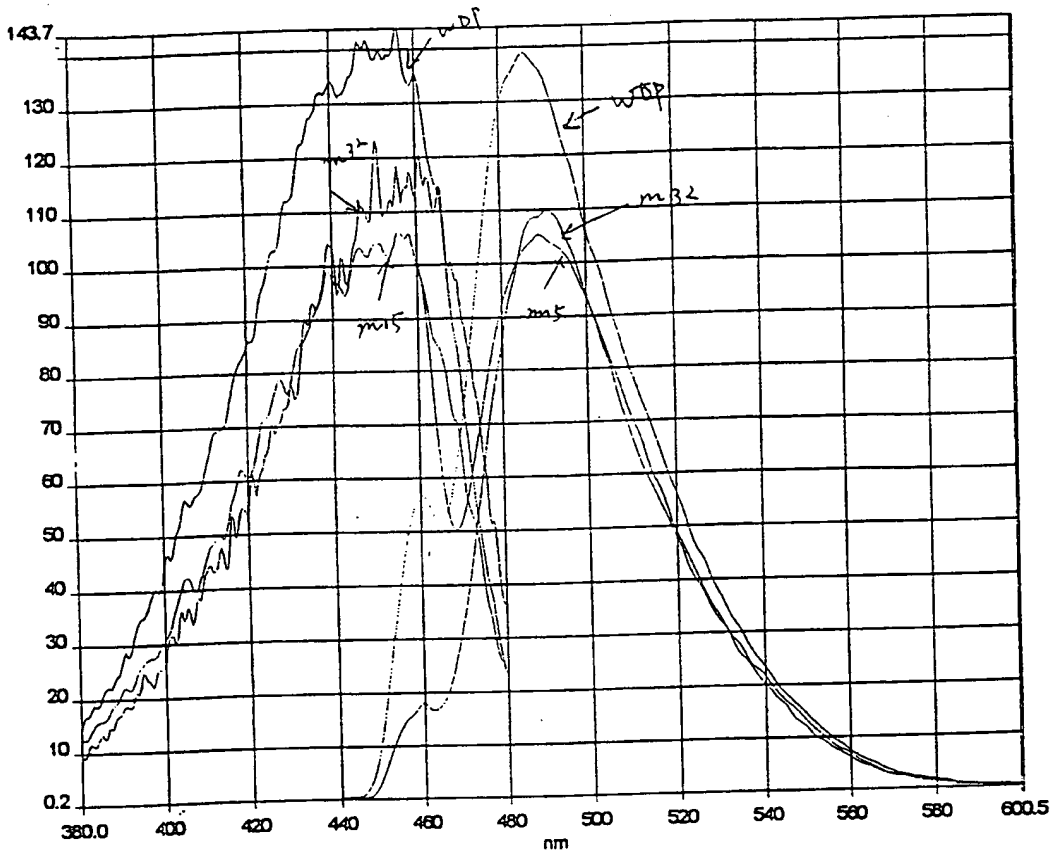


Figure 13

SEQUENCE LISTING

<110> Lukyanov, Sergey A.
Labas, Yulii A.
Matz, Mikhail V.
Fradkov, Arcady F.
Chen, Ying
Hu, Lanrong
Ding, Li
Fang, Yu

<120> Fluorescent proteins from non-bioluminescent species of Class Anthozoa, genes encoding such proteins and uses thereof

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Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys
35 40 45
Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
50 55 60
Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro
65 70 75
Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu
80 85 90
Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn
95 100 105
Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val
110 115 120
Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn
125 130 135
Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val
140 145 150
Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe
155 160 165
Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp
170 175 180
His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu
185 190 195
Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29393

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :(IPC 7): C07K 14/435; C12N 1/00, 1/15, 1/21, 5/10, 15/12, 15/63

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 252.3, 252.33, 325, 410, 254.11, 348, 369, 69.1; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
****	The sequence diskette submitted with the description was defective; thus the doscuments listed below were obtained solely by a word search. No SEQ ID NOs. could be searched.	*****
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. October 1999. Nature Biotechnology, Volume 17, No. 10, pages 969-973, entire document.	1-34
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document	24-29, 30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 FEBRUARY 2000	Date of mailing of the international search report 17 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GABRIELE ELISABETH BUGAISKY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29393

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANDERLUH et al. Cloning, sequencing and expression of equinatoxin II. 1996. Biochemical and Biophysical Research Communications. Volume 220, No. 2, pages 437-442, entire document.	1-5, 8, 12, 20-21, 23-28, 31
X	MACEK et al. Intrinsic tryptophan fluorescence of equinatoxin II, a pore-forming polypeptide from the sea anemone, Actinia equina	24-28, 31
A	L, monitors its interaction with lipid membranes. 1995. European Journal of Biochemistry, Volume 234, pages 329-335, entire document.	1-5, 8, 12, 20-21, 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29393

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/320.1, 252.3, 252.33, 325, 410, 254.11, 348, 369, 69.1; 530/350; 536/23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

dialog files 155, 5, 434, 33, 357, 35(Medline, Biosis, Scisearch, Oceanic Abs., Derwent Biotech. Abs., Dissertation Abs.); STN-CAS files Registry, CAPLUS; WEST files USPT, Derwent WPI
search terms: fluoresc? , Bioluminesc?, Protein, anthozo? , Zoanth?, Corralimorph?, Discosom?, Coral?, Alga, algae, discosom?, Cnidar?, Invert?, Rhodact?, Actinodisc?, Magenta, clavularia, zoanthus, anemonia, majano, anemon?, Zoanthar?, Actiniar?, Zoanthid?, Stolonif?, Alcyonar?, malsnkfig/sqsp, amfp486, striata, sulcata, Endomyar?